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Tropomyosin-binding properties of the CHASM protein are dependent upon its calponin homology domain

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ABSTRACT

The calponin homology-associated smooth muscle protein (CHASM) can modulate muscle contractility, and its biological action may involve an interaction with the contractile filament. In this study, we demonstrate an interaction between CHASM and tropomyosin. Deletion constructs of CHASM were generated, and pull-down assays revealed a minimal deletion construct that could bind tropomyosin. Removal of the calponin homology (CH) domain or expression of the CH domain alone did not enable binding. The interaction was characterized by microcalorimetry with a dissociation constant of 2.0×10^{-6} M. Confocal fluorescence microscopy also showed green fluorescent protein (GFP)–CHASM localization to filamentous structures within smooth muscle cells, and this targeting was dependent upon the CH domain.

Structured summary:
MINT-7966126: *CHASM* (uniprotkb:Q99LM3), *Tropomyosin alpha* (uniprotkb:P04268) and *Tropomyosin beta* (uniprotkb:P19352) *physically interact* (MI:0915) by *isothermal titration calorimetry* (MI:0065)
MINT-7966073: *CHASM* (uniprotkb:Q99LM3) *physically interacts* (MI:0914) with *Tropomyosin beta* (uniprotkb:P58776) and *Tropomyosin alpha* (uniprotkb:P58772) by *pull down* (MI:0096)
MINT-7966187: *Tropomyosin alpha* (uniprotkb:P04268) and *Tropomyosin beta* (uniprotkb:P19352) *physically interact* (MI:0915) with *CHASM* (uniprotkb:Q99LM3) by *pull down* (MI:0096)
MINT-7966090: *CHASM* (uniprotkb:Q99LM3) *binds* (MI:0407) to *Tropomyosin alpha* (uniprotkb:P04268) by *pull down* (MI:0096)

1. Introduction

The calponin homology-associated smooth muscle protein (CHASM, also known as smoothelin-like 1) is a novel member of the smoothelin family of muscle proteins [1] and is able to modulate the contractile activity of both smooth and skeletal muscle [2]. Based on alignments with the smoothelin proteins, CHASM can be divided into two separate functional regions (Fig. 1A): an N-terminal unfolded region (aa 1–361) with minimal sequence similarity to the other smoothelin proteins and a C-terminal, type-2 calponin homology (CH) domain (aa 361–459) [3]. Although CHASM showed the highest sequence similarity to smoothelins in the C-terminal type-2 CH-domain (residues 347–459) [1,3], there was also similarity within a tropomyosin-binding domain (TBD) of smoothelin that was found to be necessary for the localization of the protein to actin-containing filaments [4].

While analyses of mice lacking smoothelin isoforms have revealed critical roles for these proteins in intestinal [5] and arterial smooth muscle contractility [6], the specific biological role of CHASM in muscle remains poorly defined. The CHASM protein appears to modulate the contractile activity of smooth and striated muscles [1,2,7], and enhanced vascular and skeletal adaptations to exercise have been identified in *Smtnl1*$^{-/-}$ mice [2]. Most notably, aortic smooth muscle from *Smtnl1*$^{-/-}$ animals exhibited enhanced vasorelaxation before exercise, similar in extent to that achieved after endurance training in wild-type animals. Furthermore, the contractile response of *Smtnl1*$^{-/-}$ aortic smooth muscle to α-adrenergic agonists was greatly attenuated. Overall, these experiments

Abbreviations: CaM, calmodulin; CH, calponin homology; CHASM, calponin homology-associated smooth muscle protein; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; ITC, isothermal titration calorimetry; K_d, dissociation constant; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline; TBD, tropomyosin-binding domain; Tn, troponin

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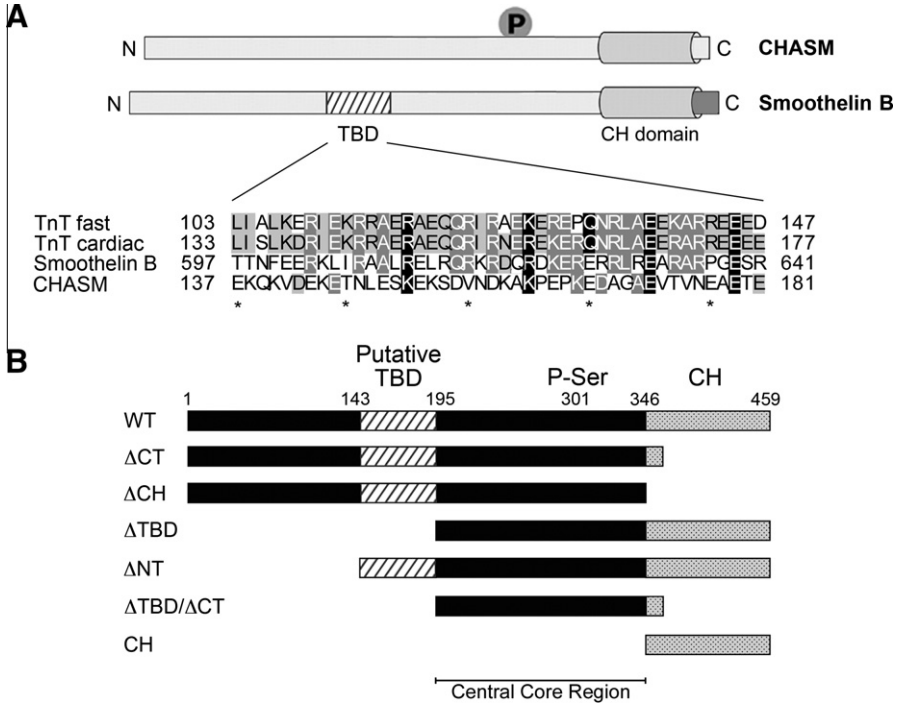

Fig. 1. The domain structure of CHASM and smoothelin proteins. (A) The tropomyosin-binding domain (TBD) of smoothelin-B is shown as a hatched box. The calponin homology (CH) domains are shown as cylinders. The C-terminal alternative splice variant region of smoothelin-B is in dark gray, and the Ser-301 phosphorylation site of CHASM as 'P'. Mouse troponin T from heart muscle (TnT cardiac, P50752) and from fast skeletal muscle (TnT fast, AAF01502) were aligned to mouse smoothelin-B (AAD29628.2) and CHASM (NP_077192) in the region surrounding the TBD. In (**B**), the CHASM truncations used to assess tropomyosin binding are illustrated. The putative tropomyosin-binding domain (TBD) of CHASM is shaded in white and the CH domain is grey.

suggest a physiological role for CHASM in the direct modulation of contractile activity.

In this study, we provide the first evidence that CHASM can bind to tropomyosin and describe the structural determinants required for the interaction. In the comparison of primary sequences between CHASM and smoothelins, we focused on the TBD region that was previously demonstrated to drive the localization of smoothelin to actin/tropomyosin-containing filaments in cells [4]. Interestingly, the interaction of CHASM with tropomyosin did not require this putative TBD sequence but instead was dependent on the presence of the complete CH domain sequence as well as a section of the disordered, "central core region" (i.e., residues 195–361). Finally, the localization of green fluorescent protein (GFP)–CHASM fusions within A7r5 smooth muscle cells reflects the tropomyosin-binding properties of CHASM and also indicate that the intracellular localization of CHASM to filamentous structures was dependent on the CH domain.

2. Materials and methods

2.1. Materials

Purified chicken gizzard tropomyosin was kindly provided by Dr. Michael Walsh (University of Calgary). An anti-CHASM antibody was provided by Dr. Timothy Haystead (Duke University). PreScission protease, glutathione-Sepharose, pGEX-6P1 and pcDNA3.1 plasmids were all purchased from GE Healthcare (Piscataway, NJ). All other chemicals were of reagent grade and purchased from VWR Scientific (Edmonton, AB) or Sigma Chemical Company (St. Louis, MO).

2.2. Cloning and expression of CHASM constructs

Various CHASM fragments (Fig. 1B) were amplified from I.M.A.G.E. clone 3593616 (Research Genetics) by standard PCR techniques with primers designed from the mouse CHASM sequence: ΔCH, bp 1–1038/aa 1–346; ΔCT, bp 1–1083/aa 1–361; ΔTBD/ΔCT, bp 583–1083/aa 195–361; ΔNT, bp 427–1377/aa 143–459; ΔTBD, bp 583–1377/aa 195–459 and CH domain, bp 1036–1377/aa 346–459. See supplementary Table 1 for a list of primers used. Glutathione-*S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21 (DE3) and isolated with glutathione-Sepharose resin. In some cases, the GST-tag was removed by cleaving 'on-column' with PreScission Protease.

2.3. Identification of tropomyosin as a CHASM binding protein in ileal smooth muscle extract

We performed affinity column-based isolations of CHASM-interacting proteins from rabbit ileum. Smooth muscle tissue was isolated, homogenized in STE buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, pH 7.2) and clarified by centrifugation. The ileal extract was incubated with GST–CHASM, GST–CHASM-ΔCH or GST proteins immobilized to glutathione-Sepharose. After extensive washing, CHASM-binding proteins were eluted with 0.5 M NaCl in STE and resolved by SDS–PAGE. Protein bands were excised from the gel and subjected to 'in-gel' trypsin digestion [8]. Extracted peptides were de novo sequenced by tandem mass spectrometry (MS/MS) and searched against the PIR1 database using the FASTS algorithm [9].

2.4. Binding of tropomyosin to immobilized GST–CHASM mutants

Further refinement of the tropomyosin-binding region was obtained with various GST–CHASM truncation proteins (Fig. 1B). GST–CHASM proteins were immobilized on glutathione-Sepharose and then incubated with purified chicken gizzard tropomyosin. After extensive washing with STE buffer containing 350 mM NaCl, CHASM-bound tropomyosin was eluted with SDS–PAGE loading

buffer and visualized by western blotting (anti-α-tropomyosin antibody; TM311, Sigma). The binding experiment was repeated using Alexa-Fluor680-labeled tropomyosin to visualize both tropomyosin isoforms. After binding and elution, the recovered fluorescent-labeled tropomyosin was subjected to SDS–PAGE, transferred onto nitrocellulose and visualized using a STORM imager.

2.5. 'Far-western' overlay of fluorescent-labeled tropomyosin with GST–CHASM mutants

The tropomyosin–CHASM interaction was also examined with a 'far-western' overlay using Alexa-Fluor680-labeled tropomyosin. The various purified CHASM mutants were separated by SDS–PAGE and transferred onto nitrocellulose. The membrane was blocked overnight in 5% (w/v) non-fat milk in phosphate-buffered saline (PBS) followed by incubation for 3 h at 4 °C with 10 μg/ml Alexa-Fluor680-labeled tropomyosin in PBS plus 5 mg/ml bovine serum albumin. After brief washing in PBS, Alexa-Fluor680-labeled tropomyosin was detected with a STORM imager.

2.6. Binding of CHASM to immobilized tropomyosin-Sepharose

Tropomyosin or Tris–HCl (as control) was covalently bound to CNBr-activated Sepharose (GE Healthcare) according to the manufacturer's instructions. Freshly purified CHASM or CHASM-ΔCT in binding buffer (25 mM Tris pH 7.2 with 0.1% (v/v) β-mercaptoethanol) was incubated with control- or tropomyosin-Sepharose for 2 h at 4 °C. After extensive washing with binding buffer plus 150 mM NaCl, bound CHASM proteins were detected by Western blotting.

2.7. Isothermal titration calorimetry (ITC) measurements

All ITC experiments were carried out on a MicroCal VP-ITC microcalorimeter over a range of temperatures (15–37 °C). Solutions of CHASM–CH, CHASM-ΔTBD/ΔCT or CHASM-ΔTBD were sequentially injected into a sample cell containing tropomyosin in ITC buffer (20 mM HEPES, pH 7.5, 50 mM KCl and 1 mM β-mercaptoethanol). The controls for the heat of dilution were obtained by titrating each CHASM protein into the ITC buffer in the absence of tropomyosin. Protein concentrations were determined using the predicted molecular extinction coefficient (ε280): CHASM-ΔTBD, 24,200 cm^{-1} M^{-1}; CHASM-ΔTBD/ΔCT, 12,490 cm^{-1} M^{-1}; CHASM–CH, 18,450 cm^{-1} M^{-1} and tropomyosin (assuming 100% α/β heterodimer), 10,430 cm^{-1} M^{-1}. The data was fit to a one-site binding model (MicroCal Origin software) after the subtraction of the heat of dilution control to obtain the dissociation constant (K_d).

2.8. Cell culture, expression of GFP–CHASM constructs and microscopy

Rat vascular smooth muscle cells (A7r5 cells, ATCC Nr. CRL-1444™) were maintained in Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen) in a humidified tissue culture incubator at 37 °C with 5% CO_2. The cells were transiently transfected at passage 15–20 with GFP–CHASM, GFP–CHASM-ΔCT or empty vector (GFP only) in pcDNA3.1 using Fugene 6 (Invitrogen). The cells were split after 2 days and re-plated onto glass coverslips. The following day, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4), mounted with ProLong Gold Antifade Media (Invitrogen) and viewed using an Olympus Fluoview 1000 laser scanning confocal microscope. Localization of GFP–CHASM, GFP–CHASM-ΔCT and GFP was demonstrated by direct fluorescence; stacks of optical sections were acquired by sequential acquisition and analyzed with FVv1.7a software. A rhodamine phalloidin-based F-actin Visualization Kit (Cytoskeleton Inc., Denver, CO) was used per the manufacturer's instructions to visualize F-actin.

3. Results

3.1. Identification of CHASM as a tropomyosin-binding protein

After capture of rabbit ileal smooth muscle proteins, a band with an approximate molecular weight of ~40 kDa was highly enriched with the GST–CHASM resin but not with the control GST column (Fig. 2). Four tryptic peptides (DSLLAADEAAAK, *m/z* 687.8, 2+; DDLEETLASAK, *m/z* 703.4, 2+; DLEEEVLQ, *m/z* 622.4, 2+ and VLLEGELE, *m/z* 585.9, 2+) were sequenced de novo with tandem MS/MS. The two best identities with expectation values of $1.6 \times e^{-6}$ and .052 were the α and β subunits of tropomyosin, respectively. The next best match had an expectation value of 3.5. Thus, from these results we concluded that CHASM could bind to the smooth muscle α/β tropomyosin heterodimer. When a CHASM construct lacking the CH domain (i.e., GST–CHASM-ΔCH) was used, a minimal amount of tropomyosin was recovered (Fig. 2). This was the first evidence to suggest that the CH domain was important for the interaction of CHASM with tropomyosin.

3.2. Tropomyosin binding to CHASM requires the CH-domain but not a degenerate TBD

An alignment of the TBDs of skeletal muscle and cardiac troponin T (TnT), smoothelin-B and CHASM confirmed that there was some minor sequence similarity in the TBD of TnT and smoothelin [4]; however, the CHASM sequence that corresponds to the smoothelin TBD is quite divergent (Fig. 1A), with only six charged residues conserved. A series of CHASM fragments (Fig. 1B) were used for in vitro binding experiments and demonstrated that tropomyosin-binding to CHASM was not dependent upon the TBD sequence (Fig. 3A) since a CHASM construct lacking this domain could still effectively interact with tropomyosin. CHASM constructs that included the central core region and the CH domain (i.e., CHASM-ΔTBL and CHASM-ΔNT) provided maximal binding; however, the CH domain in isolation was not capable of binding to tropomyosin. Preliminary data collected from ^{1}H, ^{15}N-HSQC NMR spectra of CHASM-ΔTBD suggest that many residues within α-helix-I of the CH domain are involved in intramolecular interactions with the central core region to generate structure (MacDonald and Vogel, unpublished data). So, a CHASM construct

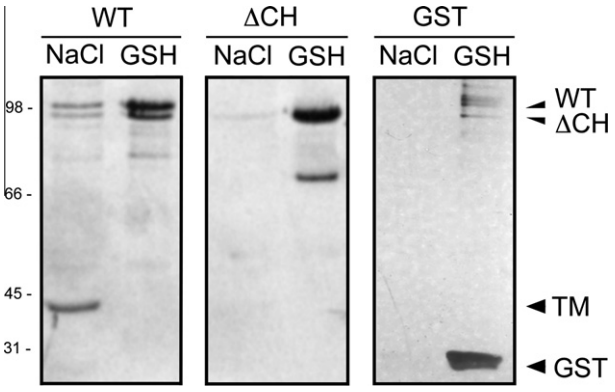

Fig. 2. CHASM binds to tropomyosin from ileum extract. GST–CHASM, GST–CHASM-ΔCH or GST were immobilized on glutathione-Sepharose resin and incubated with rabbit ileum extract; proteins bound to the various GST-fusion proteins were eluted with 0.5 M NaCl. As a loading control, glutathione (GSH, 10 mM) was applied to the columns to assess total GST-fusion protein used as 'bait'. The location of GST–CHASM (WT), GST, GST–CHASM-ΔCH and tropomyosin (TM) are indicated on the *right*, molecular weight markers are indicated on the *left*.

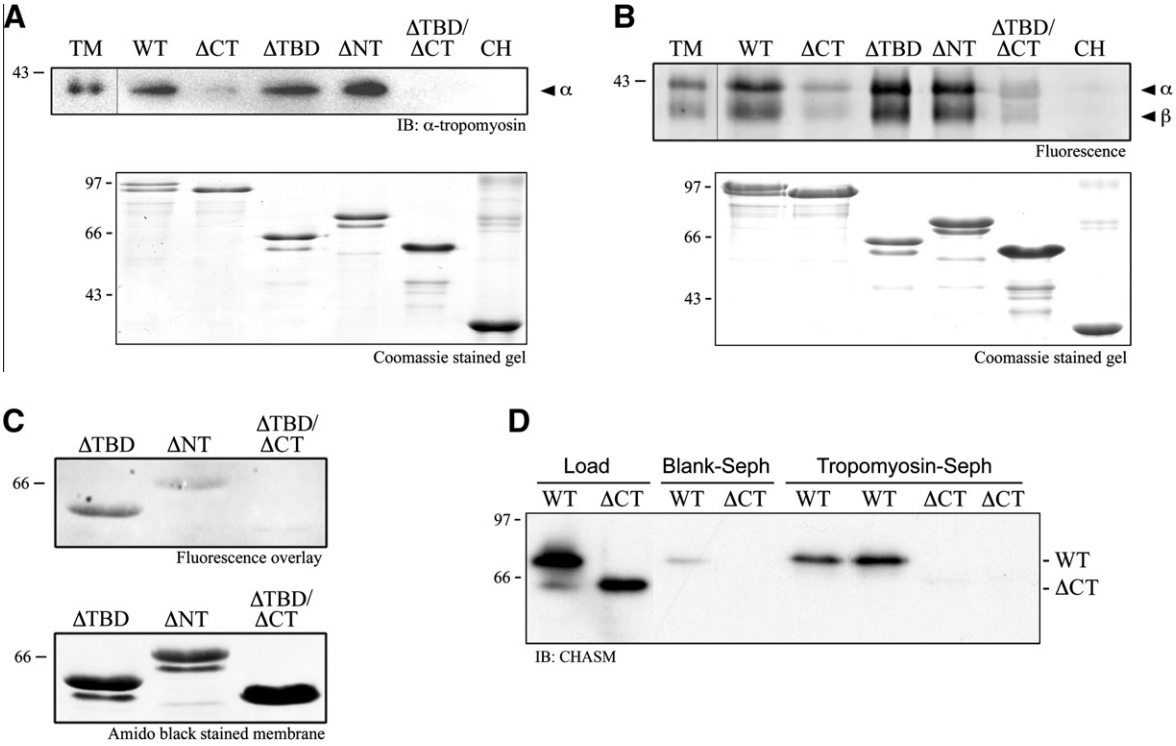

Fig. 3. CHASM binding with tropomyosin requires the complete CH domain sequence and the central core region. (A) GST-tagged, full-length CHASM (WT) or its truncation variants (100 μg) were bound to glutathione-Sepharose resin and purified chicken gizzard tropomyosin (100 μg) was added. The α isoform of tropomyosin was detected by western blotting. (B) Both α and β isoforms were detected when the pull-down was performed with fluorescent-labeled tropomyosin, followed by SDS–PAGE and transfer to nitrocellulose. Fluorescent-labeled tropomyosin was detected using a STORM imager. (C) The interaction between purified GST-tagged CHASM variants and tropomyosin (10 μg/ml) was detected by a 'far-western' overlay. (D) CHASM or CHASM-ΔCT proteins were bound to immobilized tropomyosin-Sepharose or control-Sepharose (blank). After extensive washing, bound CHASM proteins were eluted and detected by western blotting. In all cases, the images are representative of $n = 3$ separate pull-down or far-western experiments. The locations of α and β isoforms of tropomyosin are indicated on the right, molecular weight markers are on the left. Total protein was stained using Coomassie or Amido black stain to verify equal loading.

(CHASM-ΔTBD/ΔCT) that retained the α-helix-I sequence was also generated and used to assess tropomyosin-binding properties. Weak binding of tropomyosin was observed for the CHASM-ΔTBD/ΔCT constructs (Fig. 3A and B) that possessed this C-terminal deletion, suggesting that residues within the remainder of the CH domain act to stabilize the association. The experiment was repeated with fluorescent-labeled tropomyosin (Fig. 3B). Both α- and β-isoforms of tropomyosin were detected, and these results were in agreement with our initial identification of both α- and β-tropomyosin isoforms by tandem MS/MS. 'Far-western' overlays were performed with immobilized CHASM-ΔTBD, CHASM-ΔNT or CHASM-ΔTBD/ΔCT and fluorescent-labeled tropomyosin (Fig. 3C). In agreement with our previous results, tropomyosin was found to associate with CHASM-ΔTBD and CHASM-ΔNT, but not with CHASM-ΔTBD/ΔCT. Finally, immobilized α/β-tropomyosin-Sepharose was generated to explore whether CHASM could be captured using this support as bait (Fig. 3D). In this case, CHASM was readily recovered from tropomyosin-Sepharose while the partial deletion of CH domain sequence ablated binding.

3.3. ITC characterization of the tropomyosin–CHASM interaction

ITC was used to quantitatively characterize the binding of CHASM-ΔTBD to purified chicken gizzard tropomyosin (Fig. 4). The addition of CHASM-ΔTBD to tropomyosin caused an exothermic reaction with a calculated K_d of 2.0×10^{-6} M whereas the addition of CHASM-ΔTBD/ΔCT or CHASM–CH proteins did not elicit any heat of binding, suggesting either very weak or absence of binding. The findings are consistent with the results of pull-down experiments outlined in the preceding section and suggest an absence of tropomyosin-binding for CHASM constructs lacking the complete CH domain sequence.

3.4. The CH domain directs CHASM intracellular localization

Upon evaluation by confocal microscopy (Fig. 5), GFP–CHASM appeared to associate in longitudinal arrays with F-actin-containing filaments throughout the interior of the A7r5 cells and extended to the cell periphery. These results are consistent with previous reports that described the localization of other smoothelin family members to filamentous structures in mouse fibroblasts (REF52 [10] and NIH3T3 [4] cells) and primary porcine vascular smooth muscle cells [11]. In contrast, GFP–CHASM-ΔCT was found diffusely distributed throughout central regions of the cell interior with some localization to the leading edge of the cell. The lack of a filamentous fluorescence pattern in A7r5 cells expressing the CHASM-ΔCT fusion protein was in general agreement with our earlier biochemical results that suggest a disruption of tropomyosin-binding properties. As a control, GFP was expressed, and fluorescence was distributed with diffuse intensity throughout the cell interior. Western blot analysis with anti-CHASM antibody revealed expression of fusion proteins that migrated at their predicted sizes, and probing of cell lysates with an anti-GFP antibody detected only the appropriate GFP fusion proteins (data not shown).

4. Discussion

Tropomyosin has been reported to bind to many proteins with a multiplicity of structural modes [12,25]. Known binding partners

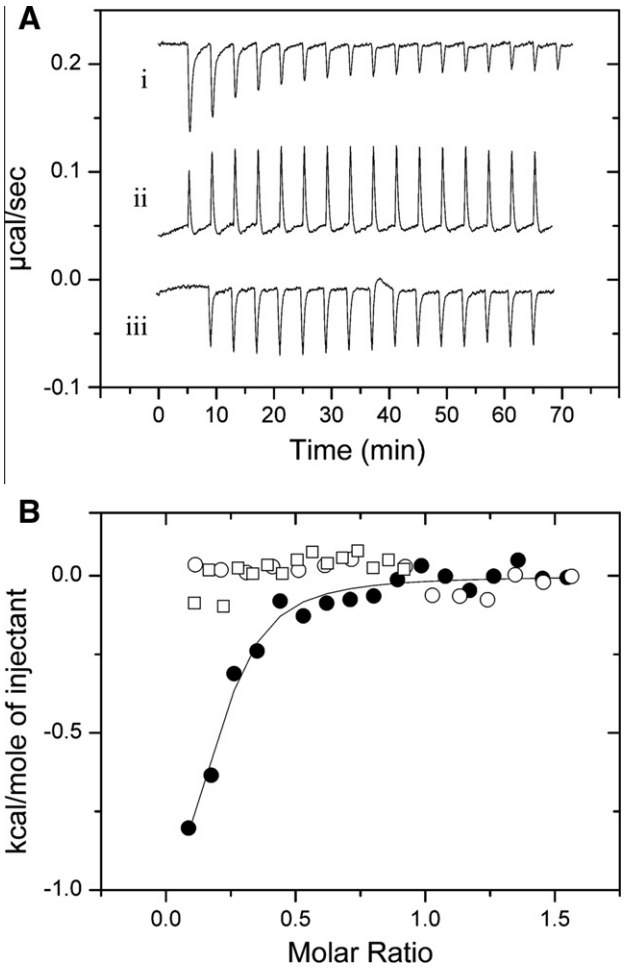

Fig. 4. Isothermal titration calorimetry reveals that CHASM binding to tropomyosin requires the complete CH domain sequence. (A) The calorimetric traces are displayed for the titrations obtained for sequential injection of 0.53 mM CHASM-ΔTBD (i), 0.34 mM CHASM-ΔTBD/ΔCT (ii) and 0.33 mM CHASM–CH (iii) into 34.5 μM tropomyosin in 20 mM HEPES buffer (pH 7.5) containing 50 mM NaCl and 1 mM β-mercaptoethanol at 25 °C. (B) The derived binding isotherms for the tropomyosin binding to CHASM-ΔTBD (●), CHASM-ΔTBD/ΔCT (□) and CHASM-CH (○) are presented.

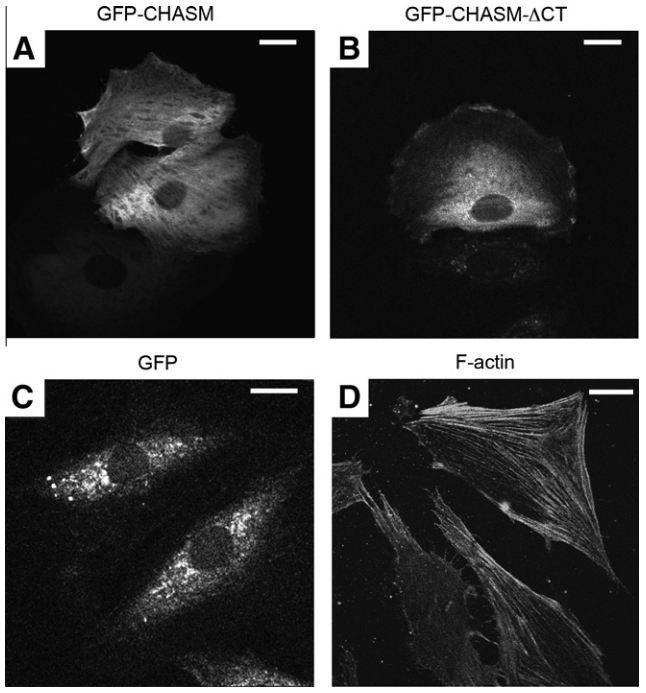

Fig. 5. Deletion of CH domain sequence alters the intracellular localization of CHASM in rat vascular smooth muscle cells. A7r5 cells were transiently transfected with pcDNA3.1 encoding GFP–CHASM (A), GFP–CHASM-ΔCT (B) or GFP (C) alone. Filamentous actin was also stained in untransfected cells with rhodamine phalloidin (D). Cells were grown on coverslips, fixed and mounted as described in Material and methods. Individual optical sections are displayed and were captured using an Olympus Fluoview 1000 laser scanning confocal microscope equipped with UPlanApo N, 60×/1.42 NA oil objective; *scale bars* = 10 μm.

include caldesmon [13], cofilin [14], calponin [15–17], S100 proteins [18,19], the LIM protein enigma [20], Rad GTPase [21], gelsolin [22] and tropomodulin [23]. Intriguingly, calponin itself was previously demonstrated to possess tropomyosin-binding characteristics [15–17]. Although the specific interaction surface was not precisely defined, tropomyosin-binding was localized to a 13 kDa chymotryptic fragment that included a type-3, N-terminal CH domain [16]. The large contact surface required for the tropomyosin interaction with CHASM is not unusual since other tropomyosin-binding proteins have binding interfaces that extend over many dozens of amino acids. For example, two TBDs identified in tropomodulin extend over the N-terminal half of the protein (residues 1–48 and 90–184; showing specificity for skeletal muscle and erythrocyte tropomyosins, respectively [23,24]). Furthermore, sequences near the boundaries of these binding regions were also deemed important for proper folding and interface of tropomodulin with tropomyosin. Our K_d value, obtained by ITC, for the binding of CHASM with smooth muscle α/β tropomyosin was in line with other previously examined tropomyosin interactions. The reported K_d value was $\sim 10^{-6}$ M for the tropomodulin association with tropomyosin [24]. In future studies, it will be important to define the location of the CHASM binding domain on tropomyosin and determine the efficiencies of CHASM binding for skeletal muscle tropomyosin since a histological examination of muscle beds demonstrated a high expression of CHASM protein in Types I and 2a striated muscle fibers [2].

Most proteins that possess CH domains can bind F-actin, yet studies have consistently concluded that a single CH domain is not sufficient for this biological property [26]. In a previous report, we have demonstrated that CHASM lacks F-actin binding characteristics in vitro [1], so the precise role of the single CH domain in CHASM was undefined. Our finding that GFP–CHASM, but not GFP–CHASM-ΔCT, was associated with filaments in cultured A7r5 smooth muscle cells suggests that the CH domain contributes to the tropomyosin-binding properties of CHASM and enables the targeting of the protein to contractile filaments. It has been previously suggested that phosphorylation-dependent changes in the biological activity of CHASM and the protein's effects on muscle contractility did not occur via direct inhibitory mechanisms but rather through interactions with the contractile filament [2]. It bears emphasis that the PKG-dependent phosphorylation site (i.e., Ser-301) is located within the putative tropomyosin-binding region of CHASM, and thus, phosphorylation could have a significant effect on the localization of the protein to thin-filaments. Future studies will be required to provide insight into the spatial localization of phosphorylated CHASM and its in vivo effects on the smooth muscle thin filament system.

We have previously demonstrated that the CH domain of CHASM possesses a calmodulin (CaM)-binding IQ-motif, and this domain could mediate an interaction with the two Ca^{2+}-binding loop regions contained within the C-domain of apo-CaM [3]. In the present study, our examination of the CHASM interaction with tropomyosin revealed that the CH domain was also required. The binding affinity of CHASM-ΔTBD for either apo-CaM or

tropomyosin is comparable; the affinity of apo-CaM for CHASM-ΔTBD ($K_d = 8.7 \times 10^{-6}$ M) was only slightly higher than that determined for tropomyosin ($K_d = 2.0 \times 10^{-6}$ M). These results imply that apo-CaM-binding may be interrupted with tropomyosin association and vice versa. However, it is also possible that the contribution of the CH-domain to apo-CaM- and/or tropomyosin-binding is not mutually exclusive. We have collected preliminary biophysical evidence that indicates deletion of the 'KTKKK' C-terminal tail abrogates apo-CaM-binding (MacDonald, unpublished data) while NMR signals originating from the 'KTKKK' tail-sequence of CHASM were not suppressed by tropomyosin binding (Ishida, H. unpublished data). Therefore, apo-CaM and tropomyosin may not share the same precise interface on the CH domain. While future studies are required to assess whether a heteromultimeric complex of CHASM, apo-CaM and tropomyosin exist in situ, our findings do suggest that the CH domain of CHASM provides a binding surface for tropomyosin as well as apo-CaM, thus placing CHASM in a unique position to influence thin-filament structure as well as Ca^{2+}-regulated contraction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.07.012.

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